

REMARKS

1. Description

1.1. The Examiner has taken the position that equations (a)-(c), as stated in claim 93, are inconsistent with the disclosure.

We agree with the Examiner vis-a-vis replacing "NAD" with --NAD⁺-- (in reaction (b)), and "NH₃" with --NH₄⁺-- in (a) and (c). Claim 93 has been so amended.

However, the Examiner is incorrect regarding the change from NAD to NADP⁺ and from NADH to NADPH in reaction (a). The Examiner refers to page 17 in connection with this rejection. The reaction set out in line 4 on page 17 is not the reaction referred to under paragraph (a) of claim 93. Rather, that is the reaction catalysed by the NADPH dependent glutamate dehydrogenase encoded by *GDH1*. That is the enzyme which is reduced or eliminated according to paragraph (ii) of claim 93. The invention in this aspect lies in replacing that enzyme activity with the enzyme activity of the enzyme GDH2 which uses NADH rather than NADPH. This is explained at page 17, lines 14 onwards: "it has been demonstrated as described herein below and in Example 1, that formation of glycerol can be reduced by deleting *GDH1* and over expressing *GDH2*". Page 17 lines 6 onwards explains that the enzyme encoded by *GDH2* catalyses the opposite reaction as that of *GADH1* but under the formation of NADH. These enzyme reactions are of course reversible and the effect of deleting or reducing activity of *GDH1* is that *GDH2* catalyses the NADH consuming reaction set out in paragraph (a) of the claim as described commencing at line 16 of page 16 "these manipulations result in a genetically modified yeast strain synthesizing glutamate under consumption of NADH rather than NADPH".

This replacement of *GDH1* with *GDH2*, its NADH-dependent isoenzyme is described in detail in connection with Example commencing on page 63.

That *GDH2* catalyses the reaction in the direction specified in paragraph (a) of the claim is made explicit at page 68, line

26 reading "as described earlier glutamate can be regenerated from 2-oxoglutamate by either of the two isoenzymes of glutamate hydrogenase, encoded by *GDH1* and *GDH2*, under consumption of NADPH or NADH respectively".

1.2. The Examiner also questions whether there is "description" for a microbial cell "of any origin", and for "any enzyme that might be able to catalyze the recited reactions".

In general, the written description requirement forbids attempts to reconceptualize the invention in a manner not set forth in the original disclosure and claims. While closely intertwined with the enablement requirement, it should not be confused with the latter. We believe that the Examiner may have confused the two.

In general, the original claims of the application necessarily satisfy the written description requirement. See, e.g., In re Koller, 204 USPQ 702 (CCPA 1980), which has never been overruled.

The PTO says that Eli Lilly identified a special set of circumstances in which the words of the claim were effectively empty of meaning, because they just were reciting a name, without any structural information, for a novel chemical compound. The PTO concluded that "in most cases", an originally filed claim is its own written description. See preamble to the January 5, 2001 written description guidelines.

In this case, the original claims recited

"microbial cells" (claim 1)

"fungal cell" (claim 6)

"yeast cell" (claim 6)

"bacterial cell" (claim 6)

We are not aware of any case that has applied the Eli Lilly doctrine outside of the context of a biomolecule (especially DNA) defined by its sequence. There is no justification for saying that a kind of cell cannot be contemplated without disclosure of (unspecified) structural information about the cell.

Nonetheless, purely because the application is under final rejection, and without prejudice or disclaimer, we have amended claim 93 to specify that the microbial cells are fungal cells. Please note that we believe that yeast cells are a kind of fungal cell, so that claims 113-116 remain properly dependent on 93. If the Examiner disagrees, we will need to amend 93 to explicitly recite yeast cells.

We turn next to the issue of enzymes from nonfungal sources. Although the description contains details only of work conducted on yeast, the biochemical pathways with which the invention is concerned are extremely fundamental ones which are common to most life forms. It is therefore a reasonable expectation that enzymes which are functionally equivalent to those described in the specification exist and regulate the same pathways universally. Furthermore, because the substrates for the enzymes are common chemicals fundamental to ammonia assimilation as a nitrogen source and because the enzymes make use of cofactors which are universal (NAD, NADH) and the universal energy source ATP/ADP, there can be no reasonable expectation that an enzyme which is functional in one species will not be functional in the same way in another.

Most organisms possess glycolysis, the pentose phosphate pathway or the citric acid pathway. Glycolysis occurs in almost every living cell and many kinds of organisms possess the pentose phosphate pathway and the citric acid cycle plays a central role in eukaryotes as well as most prokaryotes. From these pathways all of the mentioned first and second metabolites discussed in the specification are derived. For example, lactic acid is derived from pyruvate, the end product of glycolysis, via a reaction catalysed by lactate dehydrogenase. Ethanol can also be derived from pyruvate for example via a two step pathway using the reactions that are catalysed by pyruvate decarboxylase and alcohol dehydrogenase. Terpenes and sterols are derived from acetyl-CoA an intermediate of for example in the citric acid cycle. There are many more examples. All of the mentioned

pathways are redox dependent, and a change in ammonia assimilation will hence influence the production of these compounds as described in the application.

The invention of the present patent application is to apply genetic modifications to ammonia assimilation. Ammonia assimilation is highly conserved in all organisms. The introduction of inorganic nitrogen (ammonia, nitrate, nitrogen (N₂, etc.) into organic compounds proceeds always via ammonia. Attached is a drawing (Figure A) illustrating this. Nitrate, and nitrite and molecular nitrogen will always first be reduced to ammonia and only then further be metabolised into organic compounds. As can be seen from Figure A the first upper pathways describe ammonia assimilation via the GS-GOGAT pathway and glutamate dehydrogenase. It is those pathways that are subject to genetic modifications and to improvement of production of a first metabolite and to reduction of the production of a second metabolite. Hence, genetic modification in ammonia assimilation in different organisms will lead to change in the production of all relevant first and second metabolites.

Accordingly, it is certainly to be expected that the invention would be workable in different fungi cells in the same manner as described in the application.

Please find attached a print of the text of a web page of Purdue University which discusses the near universality of the two isoforms of glutamate dehydrogenase (GDH). This clearly shows that in fungi, including yeast, both GDH forms are found as a matter of generality and have been previously identified in the published art. It is also revealed that the two forms of GDH are to be found in a number of different bacteria and also in plants (Arabidopsis and Zea).

In addition, we enclose abstracts of various papers as discussed below.

Dantzig et al demonstrates the existence of the NADH-dependent glutamate hydrogenase in *Neurospora* (a fungus). Mtolera discloses the existence of the enzyme in the red alga

USSN - 09/743,414

Gratilaria sordia.

Storey et al demonstrates the existence of the enzyme in *Osteoglossum* and *Arapaima* (fish).

Dennen et al demonstrates its existence in *Schizophyllum* (a mushroom type fungus).

Lé John et al discloses the existence of the two isoenzyme GDH's in the bacterium *Thioacillus*.

The NADH dependent glutamate synthase GLT1 is equally universal. Enclosed is a web page from the European Bio Informatics Institute disclosing that it is a key enzyme in the early stages of assimilation of ammonia in bacteria, yeasts and plants. Enclosed also is an abstract by Ishiyama et al disclosing that it is present in *Oryza sativa seedlings* and in the root modules of alfalfa where it derives from a nitrogen fixing bacterium (see Vanoni et al.).

The enzyme GLN1 is equally universal. Enclosed is a copy of another web page from Purdue University regarding this and demonstrating the universality of this protein in fungi and yeasts generally.

Based upon these materials it is reasonable to conclude that the written description of the invention in the application would readily be understood by a skilled reader to be a fair exemplification of the invention as it applies across the spectrum of fungi including yeast and the skilled reader would entertain no doubt that the inventors were in full possession of the invention as claimed at the relevant date.

2. Enablement/Deposit

Claims 102, 105, 108, 111 and 115-116 refer to specific deposited biological matters, and the Examiner requires compliance with 37 CFR 1.801-1.809.

Since the specification discloses the depository accession numbers (DSM 12267, DSM 12274, DSM 12275, DSM 12276 and DSM 12277), the deposits quite obviously were made before the filing date. Hence, we don't need a "chain-of-custody" statement.

In addition, DSM is a Budapest Treaty depository, and the deposits were made under that Treaty.

Hence, we need only (and do) aver that the deposited material was accepted for deposit under the Budapest Treaty, and that all restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of the patent.

The specification has been amended to set forth the full name and address of the depository and the date of each deposit.

Respectfully submitted,

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Attorneys for Applicant

By: 

Peter P. Cooper
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Enclosures

- Dantzig et al. (abstract)
- Storey et al. (abstract)
- Dennen et al. (abstract)
- LeJohn et al. (abstract)
- EBI (web page)
- Ishiyama et al. (abstract)
- Vanoni et al. (abstract)
- Purdue (two web pages)
- Figure A
- Trepp et al. (abstract)
- Mtolera (abstract)

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Regulation of Glutamate Dehydrogenases in *nit-2* and *am* Mutants of *Neurospora crassa*

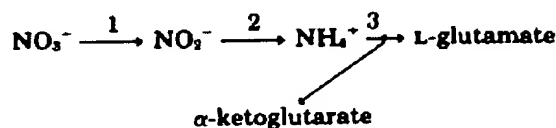
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Received for publication 27 December 1978

The regulation of the glutamate dehydrogenases was investigated in wild-type *Neurospora crassa* and two classes of mutants altered in the assimilation of inorganic nitrogen, as either nitrate or ammonium. In the wild-type strain, a high nutrient carbon concentration increased the activity of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-glutamate dehydrogenase and decreased the activity of reduced nicotinamide adenine dinucleotide (NADH)-glutamate dehydrogenase. A high nutrient nitrogen concentration had the opposite effect, increasing NADH-glutamate dehydrogenase and decreasing NADPH-glutamate dehydrogenase. The *nit-2* mutants, defective in many nitrogen-utilizing enzymes and transport systems, exhibited low enzyme activities after growth on a high sucrose concentration: NADPH-glutamate dehydrogenase activity was reduced 4-fold on NH_4Cl medium, and NADH-glutamate dehydrogenase, 20-fold on urea medium. Unlike the other affected enzymes of *nit-2*, which are present only in basal levels, the NADH-glutamate dehydrogenase activity was found to be moderately enhanced when cells were grown on a low carbon concentration. This finding suggests that the control of this enzyme in *nit-2* is hypersensitive to catabolite repression. The *am* mutants, which lack NADPH-glutamate dehydrogenase activity, possessed basal levels of NADH-glutamate dehydrogenase activity after growth on urea or L-aspartic acid media, like the wild-type strain, and possessed moderate levels (although three- to fourfold lower than the wild-type strain) on L-asparagine medium or L-aspartic acid medium containing NH_4Cl . These regulatory patterns are identical to those of the *nit-2* mutants. Thus, the two classes of mutants exhibit a common defect in NADH-glutamate dehydrogenase regulation. Double mutants of *nit-2* and *am* had lower NADH-glutamate dehydrogenase activities than either parent. A carbon metabolite is proposed to be the repressor of NADH-glutamate dehydrogenase in *N. crassa*.

Many eucaryotic microorganisms possess two distinct glutamate dehydrogenases for the interconversion of glutamate with ammonium and α -ketoglutarate, a key step in nitrogen metabolism (14). Evaluation of the factors important in the regulation of the glutamate dehydrogenases has proved difficult since the two enzymes are regulated concurrently but oppositely by both carbon and nitrogen nutrients in the medium; i.e., when one activity is high, the other is low (19, 30-33). This paper examines the regulation of the glutamate dehydrogenases in *Neurospora crassa*.

The nitrate assimilatory pathway converts inorganic nitrogen as nitrate to L-glutamate as follows:



The first two steps are catalyzed, respectively, by nitrate reductase (12, 13, 25, 26) and nitrite reductase (5, 23), and the third, by the glutamate dehydrogenases. NADPH-glutamate dehydrogenase is believed to convert NH_4^+ and α -ketoglutarate to L-glutamate, whereas NADH-glutamate dehydrogenase degrades L-glutamate (30). Each enzyme has been purified to homogeneity (3, 39).

Most mutants of the pathway have been isolated by three selection procedures: lack of growth on nitrate as a sole nitrogen source has yielded the mutants *nit-1*, *nit-2*, and *nit-3* (36); lack of growth on ammonium, the *am* mutants

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Storey, K.B., Guderley, H.E., Guppy, M. and Hochachka, P.W. 1978. Control of ammoniagenesis in the kidney of water- and air-breathing osteoglossids: control of glutamate dehydrogenase. *Can. J. Zool.* 56, 845-851.

Control of ammoniagenesis in the kidney of water- and air-breathing osteoglossids: characterization of glutamate dehydrogenase

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Abstract

Glutamate dehydrogenases (EC 1.4.1.2) from the kidney of *Osteoglossum bicirrhosum* (called aruana) and *Arapaima gigas* were kinetically characterized. The two enzymes exhibited several common characteristics including V_{max} activity ratio, pH optimum, affinity for cofactors, a marked preference for NAD(H) over NADP(H), and a very low affinity for NH₄⁺. A variety of regulatory metabolites affected both enzymes. GTP and GDP were inhibitory while ADP, ATP, AMP, and leucine activated the enzymes. Both enzymes displayed potent product inhibition which was partially reversed by low levels of ADP. *Arapaima* kidney glutamate dehydrogenase was tightly regulated by the adenylate and guanylate nucleotides, inhibition by GTP and GDP and deinhibition by ADP and AMP being much stronger for this enzyme than for the aruana enzyme. Aruana glutamate dehydrogenase, however, was more responsive to NAD-NADH control. The enzyme was more sensitive to NAD(H) product inhibition and this inhibition was poorly reversed by ADP. From these data, it was concluded that both fish kidney glutamate dehydrogenases could function in glutamate oxidation in vivo. However, the *Arapaima* enzyme appeared most clearly adapted to a catabolic roles activity being more tightly linked to the energy status of the mitochondrion. Conversely, the aruana enzyme displayed regulatory properties allowing it the potential to function in NADH oxidation during periods of hypoxic stress.

Regulation of Glutamate Dehydrogenases During Morphogenesis of *Schizophyllum commune*

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Received for publication 22 October 1966

The specific activities of two glutamate dehydrogenases (GDH), one requiring nicotinamide adenine dinucleotide (NAD) and the other specific for nicotinamide adenine dinucleotide phosphate (NADP), varied during growth of *Schizophyllum commune* as a function of the stage of the life cycle and the exogenous nitrogen source. During basidiospore germination on either glucose-NH₃ or glucose-glutamate medium, NADP-GDH increased six- to eightfold in specific activity, whereas NAD-GDH was depressed. During dikaryotic mycelial growth on either nitrogen source, the two GDH increased in a 1:1 ratio, whereas, during homokaryotic mycelial growth on glucose-NH₃, NADP-GDH activity was depressed and NAD-GDH increased six- to eightfold. Homokaryotic mycelium cultured on glucose-glutamate medium yielded high NADP-GDH activities and normal NAD-GDH activities. Intracellular NH₃ concentration and NADP-GDH activities were inversely related during spore germination and homokaryotic mycelium growth, whereas guanosine-5'-triphosphate (GTP) and L-glutamine specifically inhibited NAD- and NADP-GDH respectively in vitro. GTP inhibition was shown in extracts from cells at all stages of the life cycle. Basidiospore germling extracts contained an NADP-GDH essentially resistant to L-glutamine inhibition.

The influence of either NH₃ or L-glutamate as sole nitrogen source on the specific activity of nicotinamide adenine dinucleotide (NAD)- and NAD phosphate (NADP)-glutamate dehydrogenases (GDH) in fungi has been called coordinate regulation (15), a term which implies the simultaneous action of these metabolites or their derivatives as inducers for one enzyme and corepressors for the other. L-Glutamate has been described as a corepressor of NADP-GDH in *Neurospora crassa* (1) and *Piricularia oryzae* (8), and NH₃ has been suggested as corepressor of NAD-GDH in *Saccharomyces cerevisiae* (7). In addition, urea, one of the products of glutamate metabolism, appears to act simultaneously to induce NAD-GDH and to repress NADP-GDH in *N. crassa* (15). The repression of NAD-GDH and the induction of NADP-GDH by NH₃ in these organisms is in agreement with their cellular function documented by studies on the *am* mutants of *N. crassa* (4). In this system, NADP-GDH is anabolic (i.e., responsible for the formation of glutamate from α -ketoglutarate and NH₃), whereas NAD-GDH is catabolic and catalyzes the reverse reaction.

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The finding that NADP-GDH activity in *Schizophyllum commune* is depressed during growth of vegetative mycelium on glucose-NH₃, the reverse of that on glucose-glutamate medium, suggested that the GDH of this basidiomycete are regulated differently than in other fungal systems (3). Furthermore, the specific activities of these enzymes also changed with morphogenesis of this mushroom (D. W. Dennen and D. J. Niederpruem, *Bacteriol. Proc.*, p. 58, 1966). The present report assesses the influence of NH₃, L-glutamate, and other nitrogen sources on the activities of NAD- and NADP-GDH during the development of *S. commune* through basidiospore germination, homokaryotic mycelium growth, and dikaryotization.

MATERIALS AND METHODS

Culture conditions and strains of *S. commune*. Homokaryotic mycelium strains of *S. commune*, 699 A41B41, 845 A51B51, M1478 A41B41 lysine⁻, 70 A2B1 uracil⁻, and 667 A2B2 arginine⁻, were cultured and mated on appropriately supplemented minimal medium, and basidiospores were collected as described previously (13). A homokaryotic fruiting strain, 35hf A41B51 (Mishkin and Niederpruem, unpublished data), was used to compare spores with the routinely used dikaryotic fruit progeny. Initial

Evidence for Two Species of Glutamate Dehydrogenases in *Thiobacillus novellus*

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Received for publication 24 August 1967

When grown autotrophically in a thiosulfate-mineral salts medium, cells of the facultative chemoautotrophic bacterium, *Thiobacillus novellus*, produced two distinct glutamate dehydrogenases, one specific for nicotinamide adenine dinucleotide phosphate (NADP) and the other specific for nicotinamide adenine dinucleotide (NAD). When glutamate was supplied exogenously as the sole carbon source, the NAD-specific glutamate dehydrogenase was fully induced. Lower levels of the enzyme were found in bacteria grown in L-arginine, L-alanine, glucose, glycerol, lactate, citrate, or succinate. Arginine, histidine, and aspartate, on the other hand, caused a marked repression of the NADP-specific glutamate dehydrogenase activity. The NAD-dependent glutamate dehydrogenase was allosteric. Adenosine-5'-monophosphate and adenosine-5'-diphosphate acted as positive effectors. Both glutamate dehydrogenases were purified about 250-fold and were shown to be distinct protein with different physical properties.

It has been known for some time that the facultative chemoautotroph, *Thiobacillus novellus*, can grow rapidly on glutamate as a heterotrophic organism (10). In addition, *T. novellus* can be cultured as an autotroph in a medium composed entirely of mineral salts, when thiosulfate or some other reduced inorganic sulfur compound supplies energy and CO₂ is fixed via the Calvin-Benson pathway. In addition, glutamate can serve as a nitrogen and carbon source in a nitrogen-free medium (LéJohn, unpublished data). It is reasonable to assume that *T. novellus* possesses an active glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase EC 1.4.1.3] that can catalyze the reversible breakdown of glutamate to provide the necessary carbon and nitrogen required for growth in glutamate. It seems logical then to expect that this enzyme, which is at an important cross-link of carbohydrate and nitrogen metabolism, is regulated in some way. Either there are two enzymes, each acting independently, favoring synthesis or breakdown or, alternatively, the same enzyme is under some form of regulation.

Glutamate dehydrogenases (GDH) have been widely studied in plants, animals, and fungi. A detailed review of this appeared recently (3). There is, as yet, relatively little information available on the GDH of bacteria. A widespread belief is that bacteria possess only one type of GDH, which is either specific for nicotinamide adenine

dinucleotide (NAD) or for nicotinamide adenine dinucleotide phosphate (NADP). This report clearly shows that, in *T. novellus*, there are two distinct GDH, one specific for NAD and the other specific for NADP. The NAD-linked enzyme is inducible and allosteric (6).

MATERIALS AND METHODS

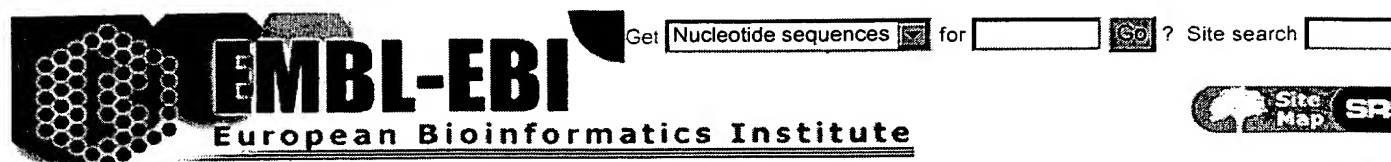
Bacteria and culture conditions. A strain of *T. novellus* (ATCC 8093) was grown and harvested as described elsewhere (7). Bacterial cells were cultured in a glutamate-thiosulfate mineral salts medium to isolate the two GDH. During induction studies, the pertinent carbon source was substituted for glutamate.

Protein determination. Protein content was determined spectrophotometrically (11) and by the phenol method of Lowry et al. (8).

Growth. Growth was determined by measuring the optical density (OD) of the cells at 530 mμ by use of a Klett-Summerson photoelectric colorimeter (the cells were first filtered through loosely packed cotton plugs to remove colloidal sulfur that is deposited during growth).

Preparation of cell-free extracts. The pellet of harvested bacteria was resuspended as a 25 to 30% (wet weight/volume) suspension of cells in 0.05 M tris-(hydroxymethyl)aminomethane chloride (Tris chloride), pH 8, containing 10⁻⁴ M reduced glutathione (GSH) and 10⁻⁴ M adenosine-5'-monophosphate (AMP) (designated TGA-buffer). The cells were disrupted in a 10-kc Raytheon sonic disintegrator (Raytheon Co., South Norwalk, Conn.) for 30 min at 5°C. Cell debris was removed by centrifugation at 48,000 × g for 15 min at 2°C. Occasionally, particulate reduced

¹ Predoctoral Fellow.



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Search:

InterPro Glutamate synthase, amidotransferase region

[?] = help

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Glu_synth_NTN

Matches: 129 proteins. View matches:

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Name [?] Glutamate synthase, amidotransferase region

Signatures [?] PF04897;Glu_synth_NTN (129 proteins)

Type [?] Domain

Dates [?] 2002-11-14 13:17:55.0 (created)
2002-11-14 13:17:55.0 (modified)

Process [?] nitrogen metabolism ([GO:0006807](#))

Function [?] glutamate synthase activity ([GO:0015930](#))

Abstract [?]

Glutamate synthase (GltS)¹ is a key enzyme in the early stages of the assimilation of ammonia in bacteria, yeasts, and plants. In bacteria, L-glutamate is involved in osmoregulation, is the precursor for other amino acids, and can be the precursor for heme biosynthesis. In plants, GltS is especially essential in the reassimilation of ammonia released by photorespiration. On the basis of the amino acid sequence and the nature of the electron donor, three different classes of GltS can be defined as follows: 1) ferredoxin-dependent GltS (Fd-GltS), 2) NADPH-dependent GltS (NADPH-GltS), and 3) NADH-dependent GltS (properties of the three classes have been reviewed extensively [1]). The enzyme is a complex iron-sulfur flavoprotein catalyzing the reductive transfer of the amido nitrogen from L-glutamine to 2-oxoglutarate to form two molecules of L-glutamate via intramolecular channeling of ammonia from the amidotransferase domain to the FMN-binding domain.

Reaction of amidotransferase domain:

$\text{L-glutamine} + \text{H}_2\text{O} = \text{L-glutamate} + \text{NH}_3$

Reactions of FMN-binding domain:

$\text{2-oxoglutarate} + \text{NH}_3 = \text{2-iminoglutarate} + \text{H}_2\text{O}$

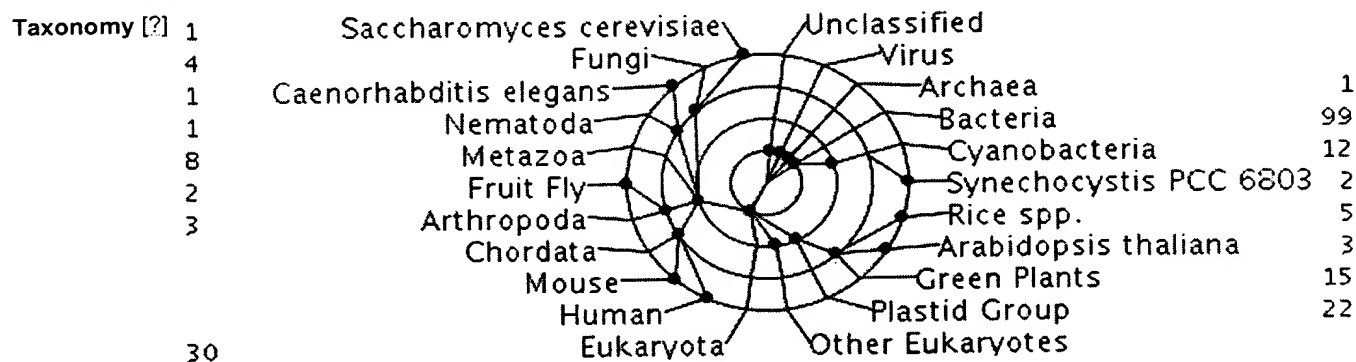
$2\text{e}^- + \text{FMN}_{\text{ox}} = \text{FMN}_{\text{red}}$

$\text{2-iminoglutarate} + \text{FMN}_{\text{red}} = \text{L-glutamate} + \text{FMN}_{\text{ox}}$

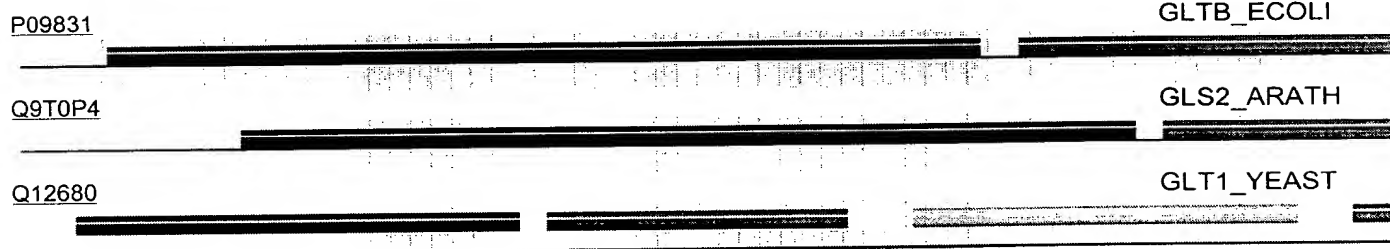
The amidotransferase domain from Fd-GltS contains the typical catalytic center of N-terminal nucleophile amidotransferases. The overall topology is characterized by a four layer alpha/beta/beta/alpha architecture, which is similar to other N-terminal nucleophile amidotransferases [2].

Structural links PDB [1ea0](#), [1llw](#), [1llz](#), [1lm1](#)
 [?] SCOP [d.153.1.1](#)

Database links
 [?] Enzyme [1.4.-.-](#)



Examples



[More proteins...](#)

- [IPR006981](#) Glutamate synthase, amidotransferase region
- [IPR000103](#) Pyridine nucleotide-disulphide oxidoreductase, class-II
- [IPR000759](#) Adrenodoxin reductase
- [IPR001327](#) FAD-dependent pyridine nucleotide-disulphide oxidoreductase
- [IPR002489](#) Glutamate synthase, alpha subunit, C-terminal
- [IPR002932](#) Ferredoxin-dependent glutamate synthase
- [IPR003009](#) FMN/related compound-binding core
- [IPR006005](#) Glutamate synthase, NADH/NADPH, small subunit 1
- [IPR006982](#) Glutamate synthase, central

Publications

1. Vanoni M.A. , Curti B.
Glutamate synthase: a complex iron-sulfur flavoprotein.
 Cell. Mol. Life Sci. 55: 617- 638 (1999) [PubMed: 10357231]

2. Van den heuvel R.H. , Ferrari D. , Bossi R.T. , Ravasio S. , Curti B. , Vanoni M.A. , Florencio F.J. , Mattevi A.
Structural studies on the synchronization of catalytic centers in glutamate synthase.
J. Biol. Chem. 277: 24579- 24583 (2002) [[PubMed: 11967268](#)]

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Expression of NADH-dependent glutamate synthase protein in epi- and exodermis of rice roots in response to the supply of nitrogen

Abstract Number: 910

Program Information

Session Number	Session Name
49	Interactions of C&N Metabolism

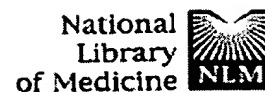
Presented by: Yamaya, Tomoyuki, tyamaya@biochem.tohoku.ac.jp

Authors:

Ishiyama, Keiki Tohoku University
Hirose, Naoya Tohoku University
Hayakawa, Toshihiko Tohoku University
Yamaya, Tomoyuki Tohoku University

The mRNA and protein for NADH-dependent glutamate synthase (NADH-GOGAT; EC 1.4.1.14) were localized in the root tips of rice (*Oryza sativa* L. cv Sasanishiki) seedlings, grown for 26 d in water alone, and markedly within 12 h of the start of a supply of a low concentration (>0.05 mM) of ammonium ions (PCP 36: 1197, 1995). This rapid response was analyzed by localizing the enzyme protein in the root tissues and by accumulation of its mRNA. When the root tips (> 1 mm) were immunostained with affinity-purified anti-NADH-GOGAT IgG, the faint signals were mainly detected in the central part of the root. Signals for Fd-GOGAT (EC 1.4.7.1) were also observed in the same area. In contrast, the root tips which were supplied with 1 mM ammonium ions for 24 h showed strong signals for NADH-GOGAT protein in two layers of the root surface, epidermis and exodermis, in addition to the central part. The supply of the ions had no effect on the profile on signals for Fd-GOGAT. Cytosolic GS was also located in those two cell layers, though the supply of ammonium ions had less effect on its localization. The results suggest that NADH-GOGAT is the limiting step for the assimilation of ammonium ions, i.e. to provide Glu for the GS reaction and that the ions absorbed are assimilated in those two-layers of cells. The mRNA for NADH-GOGAT in the whole roots accumulated maximally within 6 h of the supply of the ions, then declined. CHI had no inhibitory effect on the accumulation of the mRNA. When MSX was added together with the ammonium ions, the accumulation of the mRNA was not detected, suggesting ammonium ion itself is not a direct signal for the expression of NADH-GOGAT gene in rice roots. This work was supported by a program of Research for the Future from JSPS (JSPS-RFTF96L00604).

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Glutamate synthase: a complex iron-sulfur flavoprotein.

Vanoni MA, Curti B.

Dipartimento di Fisiologia e Biochimica Generali, Universita degli Studi di Milano, Italy.

Glutamate synthase is a complex iron-sulfur flavoprotein that forms L-glutamate from L-glutamine and 2-oxoglutarate. It participates with glutamine synthetase in ammonia assimilation processes. The known structural and biochemical properties of glutamate synthase from *Azospirillum brasilense*, a nitrogen-fixing bacterium, will be discussed in comparison to those of the ferredoxin-dependent enzyme from photosynthetic tissues and of the eukaryotic reduced pyridine nucleotide-dependent form of glutamate synthase in order to gain insight into the mechanism of the glutamate synthase reaction. Sequence analyses also revealed that the alpha subunit of bacterial glutamate synthase may be the prototype of a novel class of flavin adenine dinucleotide- and iron-sulfur-containing oxidoreductase widely used as an enzyme subunit or domain to transfer reducing equivalents from NAD(P)H to an acceptor protein or protein domain.

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Glutamate Dehydrogenase

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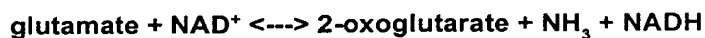
In certain fungi the main pathway of ammonia assimilation into glutamate involves NADPH-dependent glutamate dehydrogenase (GDH) [EC 1.4.1.4] (Sims and Folkes, 1951):



Direct ammonia assimilation into glutamate via the catalytic action of NADPH-GDH in the food yeast *Candida utilis* by $^{15}\text{NH}_3$ tracer experiments and quantitative analysis (Sims and Folkes, 1964).

In the filamentous fungus *Neurospora crassa* NADPH-GDH is encoded by the *gdhA* gene (Kinghorn et al, 1980). In *Aspergillus nidulans* the NADPH-GDH is designated *gdhA* (Kinghorn et al, 1986; Hawkins et al, 1989), while in *Saccharomyces cerevisiae* this gene is designated *GDH1* (Moye et al, 1985). These NADPH-GDHs are hexamers with subunit molecular weight of 60 kDa (Britton et al, 1992).

In fungi and yeasts a second NADH-dependent GDH [EC 1.4.1.2] functions primarily in catabolism (Sanwal and Lata, 1961):



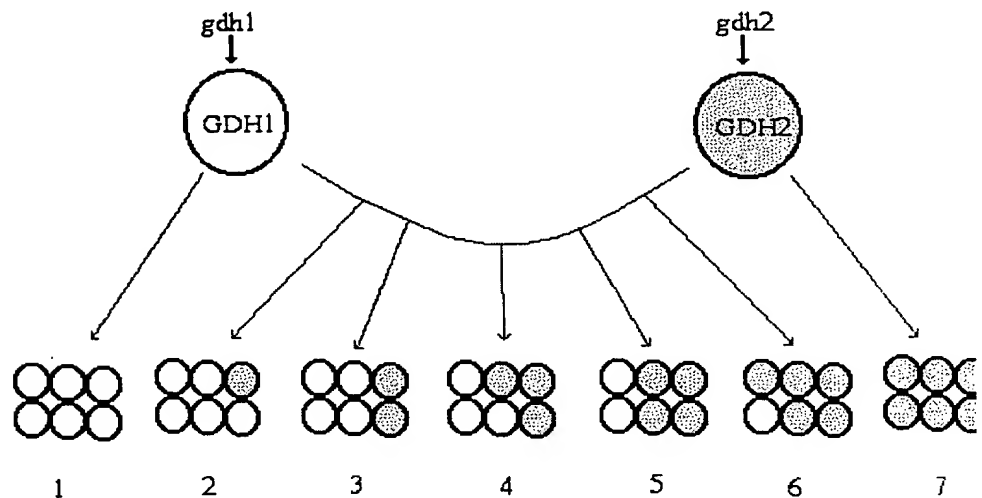
The NADH-GDH of the filamentous fungus *Neurospora crassa* is encoded by the *gdhB* gene (Kinghorn et al, 1993). The equivalent enzyme in *Aspergillus nidulans* is encoded by *gdhB* (Kinghorn et al, 1976). The NADH-GDHs of fungi and yeasts appear to be tetramers with much lower molecular weight (20 kDa) than the hexameric NADPH-GDHs involved in ammonia assimilation (Britton et al, 1992). The NADH-GDH of *Candida utilis* and *Saccharomyces cerevisiae* is regulated by reversible phosphorylation/dephosphorylation of the enzyme (Hemmings and Sirén, 1978; 1981; Uno et al, 1984). The NADH-GDH of *Neurospora crassa* is regulated by reversible phosphorylation (Kapoor et al, 1993).

Most bacteria (and cyanobacteria) possess a single NADPH-GDH isoform of the family I-type hexameric GDH proteins (Bormann et al, 1992); *Escherichia coli* [*gdhA*] (Wootton, 1983); *Salmonella typhimurium* [*gdhA*] (Miller and Brenchley, 1984); a 6803 [*gdhA*] (Chavez et al, 1995). Like the NADPH-GDHs of fungi and yeasts, the GDHs are thought to be primarily involved in ammonia assimilation rather than catabolism. However, *Bacteroides fragilis* Bf1 has two GDH activities. One is dual cofactor NAD(P)H-dependent (encoded by *gdhA*), while the other has NADH-specific activity (encoded by *gdhB*) (1998). The dual coenzyme-specific GDH is regulated by reversible inactivation in ammonium in *Bacteroides fragilis* (Yamamoto et al, 1987). In contrast to yeast, the reversible inactivation of *Bacteroides fragilis* NAD(P)H does not appear to involve phosphorylation (Yamamoto et al, 1987). The dual specific NAD(P)H-GDH of *Bacteroides theta* is typical of the family I-type hexameric GDH proteins (Baggio and Morris, 1993). The bacterium *Psychrobacter* sp. TAD1 also contains two distinct glutamate dehydrogenases specific for either NADPH or NADH (Di Fraia et al, 2000). The NADPH-GDH of *Psychrobacter* is a hexameric structure (Di Fraia et al, 2000).

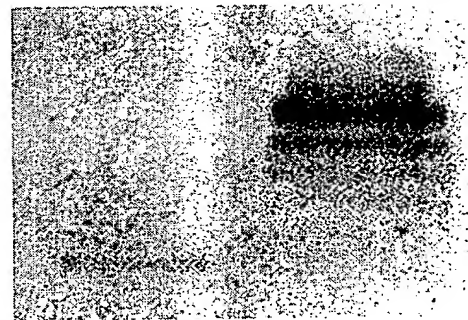
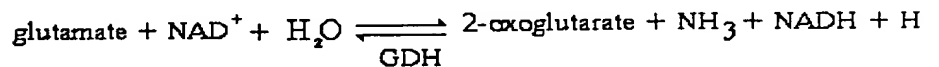
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Two distinct genes (designated *GDH1* and *GDH2*) encoding NADH-GDH have been identified in *Arabidopsis thaliana* (Turano et al, 1997). Both gene products contain putative NADH-binding polypeptides and NADH- and 2-oxoglutarate-binding domains (Turano et al, 1997). Gel filtration confirms the mitochondrial location of the NADH-GDH isoenzymes. *GDH1* encodes a 43.0 kDa polypeptide, designated alpha, and *GDH2* encodes a 43.0 kDa polypeptide, designated beta (Turano et al, 1997). The two subunits combine in different ratios to form seven GDH isoenzymes (Turano et al, 1997). In *Arabidopsis*, the slowest-migrating isoenzyme, GDH1, is a homohexamer composed of alpha subunits, and the fastest-migrating isoenzyme, GDH2, is a homohexamer composed of beta subunits (Turano et al, 1997). GDH isoenzymes are heterohexamers composed of different ratios of alpha and beta subunits (Turano et al, 1997).

Zea mays also possesses two distinct loci encoding NADH-GDH (Pryor, 1990). In the *Arabidopsis* model described above (Turano et al, 1997), maize mutants deficient in *gdh1* show only a single GDH isozyme corresponding to isozyme 7 [i.e. the homohexamer of *gdh2* gene product] (Magalhaes et al, 1990). The wildtype shows seven isozymes.



Isozymes of glutamate dehydrogenase (GDH) in plants



Native gel of GDH isozymes of the maize *gdh1*-null mutant (left) and wildtype (right) were subjected to gel electrophoresis and stained for GDH as described by Pryor (1990).

(see also discussion of GDH under gamma-aminobutyrate (GABA) metabolism in the [reactions](#) section)

In contrast to higher plants *Chlorella sorokiniana* has seven ammonium-inducible GDH isozymes composed of varying ratios of alpha- and beta-subunits (Miller et al, 1998). The *Chlorella sorokiniana* genome possesses a single 7178 bp nuclear NADPH-GDH gene (A single gene produces two 2074 and 2116 nucleotide mRNAs encoding precursors of 57,850 Da, respectively (Miller et al, 1998). The two NADPH-GDH mRNAs are in a 42 nucleotide sequence located within the 5'-coding region of the longer mRNA. This 42 nucleotide appears to undergo alternative splicing from the precursor mRNA regulated by both nutritional and environmental signals (Miller et al, 1998). The subunits of 53,501 and 52,342 Da are identical in sequence except for an 11 amino acid N-terminus of the alpha-subunit (Miller et al, 1998).

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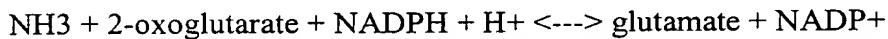
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Ammonia Assimilation and Recycling

Glutamate Dehydrogenase

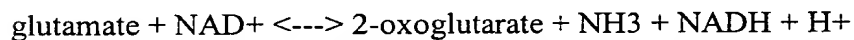
In certain fungi the main pathway of ammonia assimilation into glutamate involves the catalytic activity of NADPH-dependent glutamate dehydrogenase (GDH) [EC 1.4.1.4] (Sims and Folkes, 1964; Fincham, 1951):



Direct ammonia assimilation into glutamate via the catalytic action of NADPH-GDH was demonstrated in the food yeast *Candida utilis* by $^{15}\text{NH}_3$ tracer experiments and quantitative analysis of metabolic fluxes (Sims and Folkes, 1964).

In the filamentous fungus *Neurospora crassa* NADPH-GDH is encoded by the *am* gene (Kinsey et al, 1980). In *Aspergillus nidulans* the NADPH-GDH is designated *gdhA* (Kinghorn and Pateman, 1975; Gurr et al, 1986; Hawkins et al, 1989), while in *Saccharomyces cerevisiae* this gene is designated *GDH1* (Moye et al, 1985). These NADPH-GDHs are hexamers with subunit molecular weights of ~ 50 kDa (Britton et al, 1992).

In fungi and yeasts a second NADH-dependent GDH [EC 1.4.1.2] functions primarily in glutamate catabolism (Sanwal and Lata, 1961):



The NADH-GDH of the filamentous fungus *Neurospora crassa* is encoded by the gene *gdh* (Kapoor et al, 1993). The equivalent enzyme in *Aspergillus nidulans* is encoded by *gdhB* (Kinghorn and Pateman, 1976). The NADH-GDHs of fungi and yeasts appear to be tetramers with much larger subunit size (~ 115 kDa) than the hexameric NADPH-GDHs involved in ammonia assimilation (Britton et al, 1992). The NADH-GDH of *Candida utilis* and *Saccharomyces cerevisiae* is regulated by reversible deactivation involving phosphorylation/dephosphorylation of the enzyme (Hemmings and Sims, 1977; Hemmings, 1978; 1981; Uno et al, 1984). The NADH-GDH of *Neurospora crassa* is regulated by catabolite repression (Kapoor et al, 1993).

Most bacteria (and cyanobacteria) possess a single NADPH-GDH isoform of the hexameric type: see e.g. *Corynebacterium glutamicum* [*gdh*] (Bormann et al, 1992); *Escherichia coli* [*gdhA*] (McPherson and Wootton, 1983); *Salmonella typhimurium* [*gdhA*] (Miller and Brenchley, 1984); and *Synechocystis* PCC 6803 [*gdhA*] (Chavez et al, 1995). Like the NADPH-GDHs of fungi and yeasts, these bacterial NADPH-GDHs are thought to be primarily involved in ammonia assimilation rather than glutamate catabolism. However, *Bacteroides fragilis* Bfl has two GDH activities. One is dual

cofactor NAD(P)H-dependent (encoded by *gdhA*), while the other has NADH-specific activity (encoded by *gdhB*) (Abrahams and Abratt, 1998). The dual coenzyme-specific GDH is regulated by reversible inactivation in vivo and repression by ammonium in *Bacteroides fragilis* (Yamamoto et al, 1987). In contrast to yeast, the mechanism of reversible inactivation of *Bacteroides fragilis* NAD(P)H does not appear to involve phosphorylation (Yamamoto et al, 1987). The dual specific NAD(P)H-GDH of *Bacteroides thetaiotaomicron* encoded by *gdhA* is typical of the family I-type hexameric GDH proteins (Baggio and Morrison, 1996). The Antarctic bacterium *Psychrobacter* sp. TAD1 also contains two distinct glutamate dehydrogenases (GDH), each specific for either NADPH or NADH (Di Fraia et al, 2000). The NADPH-GDH of *Psychrobacter* has a hexameric structure (Di Fraia et al, 2000).

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Native gel of GDH isozymes of the maize *gdh1*-null mutant (left) and wildtype (right). Root extracts were subjected to gel electrophoresis and stained for GDH as described by Magalhaes et al (1990).

(see also discussion of GDH under gamma-aminobutyrate (GABA) metabolism in the Amino transferase reactions section)

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2116 nucleotide mRNAs encoding precursor proteins of 56,350 and 57,850 Da, respectively (Miller et al, 1998). The two NADPH-GDH mRNAs are identical with the exception of a 42 nucleotide sequence located within the 5'-coding region of the longer mRNA (Miller et al, 1998). This 42 nucleotide appears to undergo alternative splicing from the precursor mRNA by a process that is regulated by both nutritional and environmental signals (Miller et al, 1998). The mature alpha- and beta-subunits of 53,501 and 52,342 Da are identical in sequence except for an 11 amino acid extension at the N-terminus of the alpha-subunit (Miller et al, 1998).

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- GS is encoded by the gene *GLN1* in *Saccharomyces cerevisiae* (Mitchell, 1985).
- GS is regulated by repression/derepression (mediated by glutamine). Mutants of the yeast *Saccharomyces cerevisiae* have been isolated which fail to derepress glutamine synthetase upon glutamine limitation. The mutations define a single nuclear gene, *GLN3* (Mitchell and Magasanik, 1984b). The elevated NAD-GDH activity normally found in glutamate-grown cells is not found in *gln3* mutants (Mitchell and Magasanik, 1984b). Glutamine limitation of *gln1* structural mutants has the opposite effect, causing elevated levels of NAD-GDH even in the presence of ammonia (Mitchell and Magasanik, 1984b). A regulatory circuit that responds to glutamine availability through the *GLN3* product has been proposed (Mitchell and Magasanik, 1984b).
- Mitchell and Magasanik (1984c) propose that production of GS in *Saccharomyces cerevisiae* is controlled by three regulatory systems. One system responds to glutamine levels and depends on the positively acting *GLN3* product (Mitchell and Magasanik, 1984c). The second system is general amino acid control, which couples derepression of a variety of biosynthetic enzymes to starvation for many single amino acids (Mitchell and Magasanik, 1984c). This system operates through the positive regulatory element *GCN4*. A third system responds to purine limitation (Mitchell and Magasanik, 1984c).
- It is now known that in *Saccharomyces cerevisiae*, the transcription factors Gln3p and Nil1p of the GATA family play a determinant role in expression of genes that are subject to nitrogen catabolite repression (Soussi-Boudekou and Andre, 1999). In addition, the yeast mutant, *gan1-1*, exhibits dramatically decreased NAD-GDH and GS activities (Soussi-Boudekou and Andre, 1999). The *GAN1* gene encodes a 488-amino-acid polypeptide bearing no typical DNA binding domain (Soussi-Boudekou and Andre, 1999). Gan1p is required for full expression of *GLN1*, *GDH2* and also other nitrogen utilization genes, including *GAP1*, *PUT4*, *MEP2* and *GDH1* (Soussi-Boudekou and Andre, 1999).
- In the food yeast *Candida utilis* GS is subject to cumulative feedback inhibition by end-products of glutamine metabolism *in vitro*, but this regulation was not demonstrable *in vivo* by direct measurements of the rate of glutamine synthesis (Sims and Ferguson, 1974).
- In *Candida utilis*, GS is regulated by glutamine-mediated repression and reversible deactivation involving dissociation of active octomers into deactive tetramers (Ferguson and Sims, 1971; 1974b; Sims et al, 1974a). Sims and coworkers demonstrated a rapid inactivation of GS in *Candida utilis* on the addition of ammonia to glutamate-grown cultures. An increase in glutamine and a decrease in 2-oxoglutarate is implicated in this control (cf. glutamine/2-oxoglutarate ratio involvement in control of GS adenylation/deadenylation in gram-negative bacteria) (Ferguson and Sims, 1971; 1974b; Sims et al, 1974a). High glutamine concentrations promote the "relaxation" of the native 15.4 S enzyme into a 14.2 S octamer which dissociates reversibly into two 8.7 S tetramers. PEP promotes relaxation and formation of enzyme tetramers.

NAD⁺, NADPH and ATP cause dissociation of tetramers into monomers. Glutamate and Mg²⁺ prevent dissociation and promote reassociation of tetramers (Sims et al, 1974b). Whereas 2-oxoglutarate can prevent dissociation of octamers it cannot promote reassociation (Sims et al, 1974b). *Candida utilis* GS tetramers have the same transferase activity as octamers, but have reduced synthetase activity. In the presence of 2-oxoglutarate and glutamate the enzyme can maintain its structural integrity under conditions which would otherwise lead to dissociation (Sims et al, 1974b).

- In *Saccharomyces cerevisiae* GS is modulated by nitrogen repression and by two distinct inactivation processes (Legrain et al, 1982). Addition of glutamine to exponentially grown yeast leads to rapid enzyme inactivation that is reversed by removing glutamine from the growth medium (Legrain et al, 1982). A regulatory mutation (*gdhCR* mutation) suppresses this inactivation by glutamine in addition to its derepressing effect on enzymes involved in nitrogen catabolism (Legrain et al, 1982). The *gdhCR* mutation also increases the level of proteinase B in exponentially grown yeast (Legrain et al, 1982). Inactivation of GS is also observed during nitrogen starvation (Legrain et al, 1982). This inactivation is irreversible and consists very probably of a proteolytic degradation. Strains bearing proteinase A, B and C mutations are no longer inactivated under nitrogen starvation (Legrain et al, 1982).
- The reversible *in vivo* inactivation of *Saccharomyces cerevisiae* GS by the addition of glutamine or ammonia is characterized by a specific loss of synthetase activity; transferase activity remains stable (Mitchell and Magasanik, 1984b). Several physiological perturbations cause inactivation, such as carbon starvation or limitation for a required amino acid, which could cause a buildup of glutamine (Mitchell and Magasanik, 1984b). In contrast to *Candida utilis*, no change in the native size of the enzyme was associated with inactivation of *Saccharomyces cerevisiae* GS, but there appears to be a change in the immunological properties of the enzyme subunit (Mitchell and Magasanik, 1984b).
- *Neurospora crassa* GS is unusual in that it contains two non-identical polypeptides (Sanchez et al, 1980). When *Neurospora crassa* is grown exponentially on ammonium excess, ammonium is fixed by a glutamate dehydrogenase and an octameric glutamine synthetase (GS) (Lara et al, 1982). The synthesis of this GS polypeptide (beta) is regulated by the nitrogen source present in the medium; high on glutamate, intermediate on ammonium, and low on glutamine (Lara et al, 1982). However, when *N. crassa* is grown in fed-batch ammonium-limited cultures a different polypeptide of GS (alpha), arranged as a tetramer, is synthesized (Dunn-Coleman and Garrett, 1980; Lara et al, 1982). The tetrameric alpha GS is proposed to function with glutamate synthase in the assimilation of low ammonium concentrations (Lara et al, 1982). *gln-1b* mutant strains synthesize only the GS alpha monomer (i.e. lacks the GS beta monomer) (Calderon et al, 1990).

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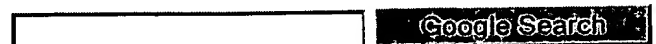
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NADH-Glutamate Synthase in Alfalfa Root Nodules. Immunocytochemical Localization¹

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ABSTRACT

In root nodules of alfalfa (*Medicago sativa* L.), N₂ is reduced to NH₄⁺ in the bacteroid by the nitrogenase enzyme and then released into the plant cytosol. The NH₄⁺ is then assimilated by the combined action of glutamine synthetase (EC 6.3.1.2) and NADH-dependent Glu synthase (NADH-GOGAT; EC 1.4.1.14) into glutamine and Glu. The alfalfa nodule NADH-GOGAT protein has a 101-amino acid presequence, but the subcellular location of the protein is unknown. Using immunocytochemical localization, we determined first that the NADH-GOGAT protein is found throughout the infected cell region of both 19- and 33-d-old nodules. Second, in alfalfa root nodules NADH-GOGAT is localized predominantly to the amyloplast of infected cells. This finding, together with earlier localization and fractionation studies, indicates that in alfalfa the infected cells are the main location for the initial assimilation of fixed N₂.

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Some Properties of Glutamate Dehydrogenase from the Marine Red Alga *Gracilaria sordida* (Harv.) W. Nelson

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Abstract

Keywords: ammonia assimilation, glutamate dehydrogenase, GDH, *Gracilaria sordida*, red alga, enzyme activity

Glutamate dehydrogenases (GDH, EC 1.4.1.2-4) catalyse the entry of ammonium into the organic cycle via amination of α -ketoglutarate and its release via deamination of L-glutamate. NAD-GDH facilitates NAD-/NADP-dependent deamination while NADPH-GDH facilitates NADH-/NADPH-dependent amination reactions. In the study reported here, GDHs were extracted from the red alga *Gracilaria sordida* and purified 10- to 180-fold before examining their amination and deamination reaction properties. NAD-/NADP- and NADH-/NADPH-dependent activities were the order of 11:1 and 1:1.8, respectively. The pH optima for amination and deamination were 8.2 and 8.4 under NADH- and NADPH- and 8.4 and 9 under NAD- and NADP- dependent activities, respectively. Whereas both NAD- and NADP-dependent deamination activities were activated by calcium ions (Ca^{2+}), only NADPH-dependent amination was activated. The K_m values (in mM) were 3.0–3.6 for ammonia, 2.0–3.3 for α -ketoglutarate, 0.00286 for NADH, 0.0033 for NADPH, 1.7–2.1 for L-glutamate, 0.344 for NAD and 0.476 for NADP. It appears that the GDHs in *G. sordida* are dominated by NAD-dependent deamination enzyme and that in NADH-NADPH- dependent amination reactions, NADPH is more preferred. It is suggested that NAD-GDH's role in *G. sordida* could be as a catabolic shunt facilitating respiration. Anabolic functions could be assimilation of ammonia released during photorespiration and synthesis of N-rich transport compounds.

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